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14. ABSTRACT

Dendritic cells (DC) have become particularly attractive candidates for cancer immunotherapy due to their potent ability to stimulate antigen specific T cells responses. A number of pre-clinical and clinical studies using tumor antigen-pulsed DCs to treat a variety of malignancies have demonstrated that DC vaccines can elicit measurable cellular anti-tumor immunity. However, despite these encouraging results, DC-based immunotherapy has demonstrated only limited clinical success in the treatment of established tumors. The limited clinical efficacy of existing DC-based cancer vaccines has been attributed in part to suppressive factors produced by the growing tumor, such as transforming growth factor-beta (TGF-β) that has been shown to impair the immunostimulatory capacity of DCs. Therefore, strategies to neutralize the deleterious effects of TGF-β may lead to more effective DC-based cancer therapies. HTS466284 and SM16 are potent small molecule TGF-β receptor type I (TβRI) kinase inhibitors that have been shown to block TGF-β signaling by binding to the ATP-binding pocket of this receptor. The hypothesis to be tested is that TβRI kinase inhibitor therapy will enhance the efficacy of DC vaccines in the treatment of established murine mammary tumors by rendering DCs resistant to TGF-β-mediated immunosuppression. The specific aims of the project are to: 1) Determine the effect of TβRI kinase inhibitors on spontaneous tumor metastasis, 2) Evaluate the effect of the combination TβRI kinase inhibitors plus DC vaccination on the treatment of primary and metastatic breast cancer, 3) Evaluate the role of immune effector cells in the anti-tumor response following combination therapy with TβRI kinase inhibitors and DC vaccines.

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INTRODUCTION

Dendritic cells (DC) have become particularly attractive candidates for cancer immunotherapy due to their potent ability to stimulate antigen specific T cells responses. A number of pre-clinical and clinical studies using tumor antigen-pulsed DCs to treat a variety of malignancies have demonstrated that DC vaccines can elicit measurable cellular anti-tumor immunity. However, despite these encouraging results, DC-based immunotherapy has demonstrated only marginal clinical success in the treatment of established tumors in cancer patients. These limitations provide rationale for investigating new strategies to augment the efficacy of existing DC-based cancer vaccines. The limited clinical efficacy of DC-based cancer vaccines has been attributed in part to suppressive factors produced by the growing tumor, such as transforming growth factor-beta (TGF-β). Therefore, strategies to neutralize the deleterious effects of TGF-β may lead to more effective DC-based cancer therapies. HTS466284 and SM16 are members of a new class of small molecule TGF-β signaling antagonists that block TGF-β signaling by selectively inhibiting the kinase activity of the TGF-β receptor type I (TβRI). The goal of this study is to use TβRI inhibitors to enhance the effectiveness of DC-based cancer vaccines in the treatment of established and metastatic TGF-β-producing murine mammary tumors. The hypothesis to be tested is that TβRI inhibitor therapy will enhance the efficacy of DC vaccines in the treatment of murine mammary tumors by rendering DCs resistant to TGF-β-mediated immunosuppression. The specific aims of this study are to: 1) Determine the effect of TβRI kinase inhibitors on spontaneous tumor metastasis, 2) Evaluate the effect of the combination TβRI kinase inhibitors plus DC vaccination on the treatment of primary and metastatic breast cancer, 3) Evaluate the role of immune effector cells in the anti-tumor response following combination therapy with TβRI kinase inhibitors and DC vaccines.

RESULTS

1. HTS466284 treatment inhibits TGF-β signaling in 4T1 tumor tissue *in vivo*.

An early event in the TGF-β signaling cascade is the phosphorylation of the transcription factor Smad2 by TβRI. Therefore, in order to determine if the TβRI kinase inhibitor HTS466284 can block TGF-β signaling *in vivo* mice bearing 21-day established 4T1 tumors were treated with 45 mg/kg of this drug for three days and their tumors were analyzed for phosphorylated Smad2 (pSmad2) by Western blot analysis. The data show that 4T1 tumors from animals treated with HTS466284 at a dose of 45 mg/kg demonstrated significantly reduced levels of pSmad2 compared to tumors from animals treated with the vehicle (DMSO) alone (Figure 1). Total Smad2 levels were unaffected by this treatment.

Figure 1. Effect of HTS466284 on TGF-β signaling in 4T1 tumor tissue in vivo. Mice bearing 21-day 4T1 tumors (volume ~107 mm^3) were injected i.p. with 45mg/kg HTS466284 or DMSO (vehicle control) for three consecutive days. One hr after the 3rd injection the tumors were homogenized, protein (35μg) was resolved by 10% SDS-PAGE, and transferred to PVDF membrane. pSmad2 was detected by incubating the membrane in rabbit anti-human pSmad2 antibody followed by HRP-conjugated goat anti-rabbit antibody. The membranes were then stripped and incubated with mouse anti-Smad2/3 antibody and membrane-bound total Smad2/3 was visualized with goat anti-mouse-HRP antibody.

2. The ability of HTS466284 to enhance the efficacy of DC vaccines in the treatment of 4T1 tumors is inconsistent.

Since previous work in our laboratory has demonstrated that tumor-derived TGF-β can impair a number of critical DC immunostimulatory functions (1)**,** we next wanted to determine if HTS466284 could enhance the efficacy of DC vaccines in the treatment of established, TGF-β-producing 4T1 tumors by inhibiting TGF-β-mediated immunosuppression. To this end mice bearing 10-day established 4T1 tumors (~19mm^3) were injected intraperitoneally (i.p.) with 45 mg/kg of HTS466284 daily for a total of 19 days. The mice were also injected subcutaneously (s.c.) with 10^6 4T1 tumor lysate-pulsed, TNF-α matured DCs on days 12, 17 and 22. Tumor volume was monitored every other day by measuring the tumor with calipers. The data indicate that treatment with the combination of HTS466284+DC significantly inhibited primary tumor growth compared to control treatments (Figure 2A). Tumors from mice in the combination therapy group grew at a significantly slower rate than tumors from animals treated with DMSO alone (P=0.01645), HTS466284 alone (P=0.00786), or DC alone (P=0.0106). The mean tumor volumes on Day 28 post-tumor injection in mice treated with DMSO, HTS466284, and DC alone were 1036.6 ± 321.4 mm^3, 1128 ± 319.4 mm^3, and 1001.4 ± 322.3 mm^3 respectively. In contrast, the mean tumor volume in mice receiving the combination of HTS466284+DC on the corresponding day was 358.1 ± 151.6 mm^3, approximately 3 times smaller. In addition, the combination treatment regimen significantly prolonged survival in these animals compared to mice treated with DMSO alone (P=0.0292), HTS466284 alone (P=0.0155), and DC alone (P=0.0254) (Figure 2B). The median survival time was increased from 30.5 days in animals treated with HTS466284 alone and 33 days in animals receiving DMSO or DC alone to 47 days in mice receiving the combination therapy. All of the mice treated with DMSO or HTS466284 alone died from large tumor burden by Day 39. All animals treated with DC alone died or were sacrificed when tumor volumes reached 1500 mm^3 by Day 44. Remarkably, two mice in the HTS466284+DC combination therapy group survived until Day 52 and Day 63 respectively. HTS466284 at 45 mg/kg was well tolerated and none of the animals displayed any overt signs of drug-related toxicity.

Figure 2A

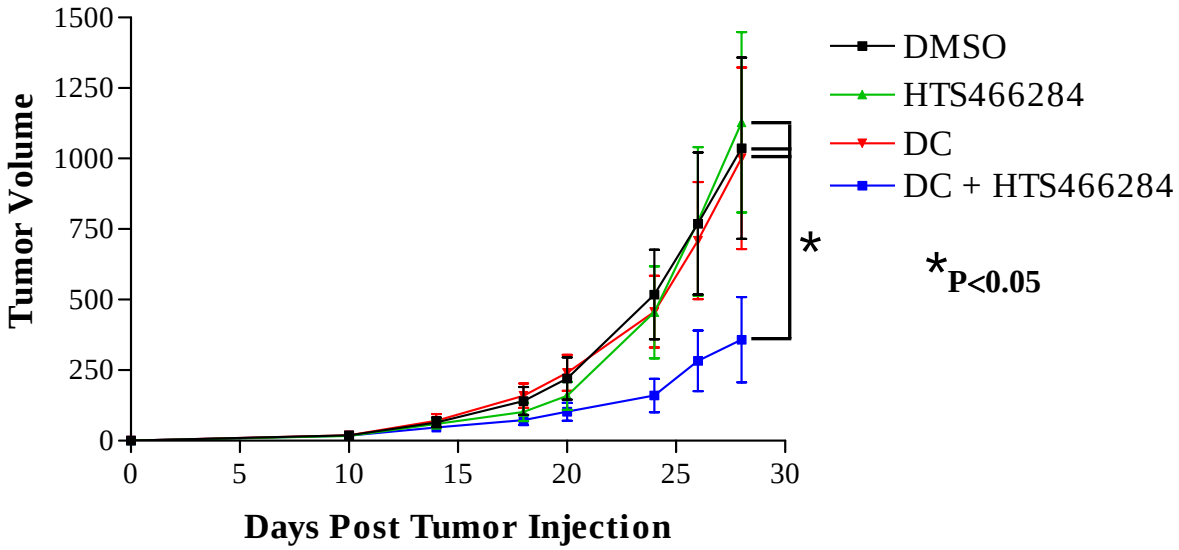

Figure 2B

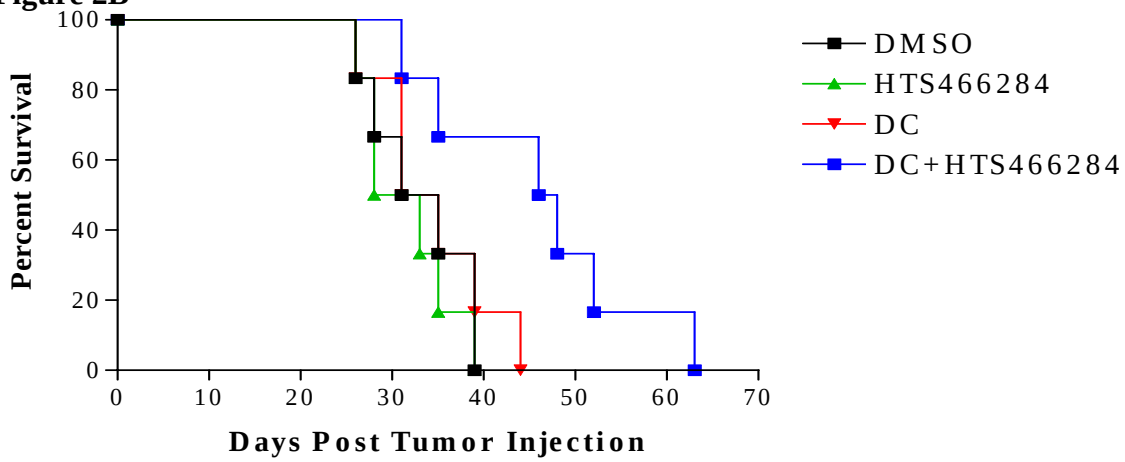

Figure 2. Effect of HTS466284 and DC vaccination on established 4T1 tumors. Six-week-old female BALB/c mice were injected s.c. on day 0 with $5x10^4$ 4T1 cells into the mammary fat pad. On day 10 when tumors reached a size of ~19 mm^3, the mice were injected i.p. with 45 mg/kg of HTS466284 in 50 µl of DMSO daily for 19 days. Control animals received 19 daily injections of DMSO alone. Mice in the DC groups were injected s.c. contralateral to the tumor site with $1x10^6$ 4T1 tumor lysate pulsed, TNF-α matured DCs on days 12, 17, and 22. The data represent: **A.** Mean tumor volume ± SEM of 6-7 individual mice and **B.** Percent survival of mice in each group. Animals either died naturally from tumor burden or were sacrificed when tumors reached an average size greater than 1500 mm^3.

Since TGF-β is known to promote tumor metastasis and since preliminary work in our laboratory demonstrated that HTS466284 inhibits 4T1 migration *in vitro*, we conducted a follow-up experiment to determine if HTS466284 therapy could improve the ability of DC vaccines to suppress the formation of spontaneous tumor metastases. For this purpose mice bearing 10-day established 4T1 tumors were injected i.p. with 45 mg/kg of HTS466284 daily for a total of 19 days as described earlier. The mice were also injected s.c. with 10^6 4T1 tumor lysate-pulsed, TNF-α matured DCs on days 12, 17 and 22. The tumors were measured as described above. In addition, 29 days post-tumor injection the animals were sacrificed and the lungs were collected and stained with India ink and the pulmonary metastases were counted visually. In this study we were unable to replicate the results from our earlier established disease study. The data show that unlike what was seen in our initial combination therapy *in vivo* experiment, HTS466284+DC had no statistically significant effect on primary 4T1 tumor growth (Figure 3A). Furthermore, the combination of HTS466284+DC failed to significantly suppress the formation of spontaneous pulmonary 4T1 metastases (Figure 3B).

Figure 3A

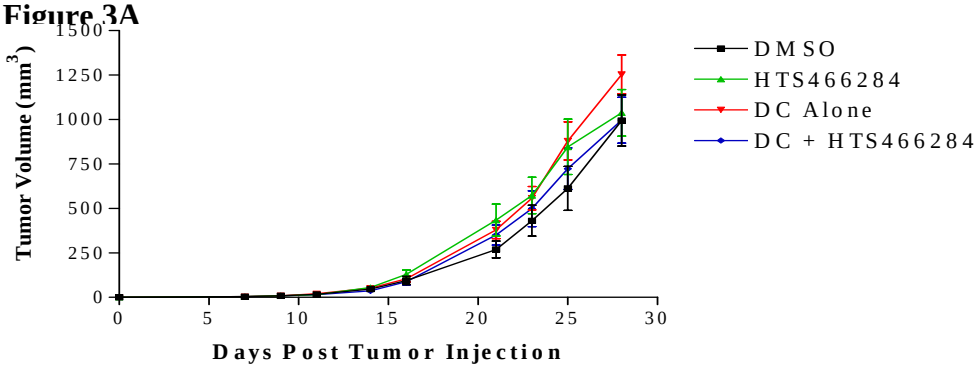

Figure 3B

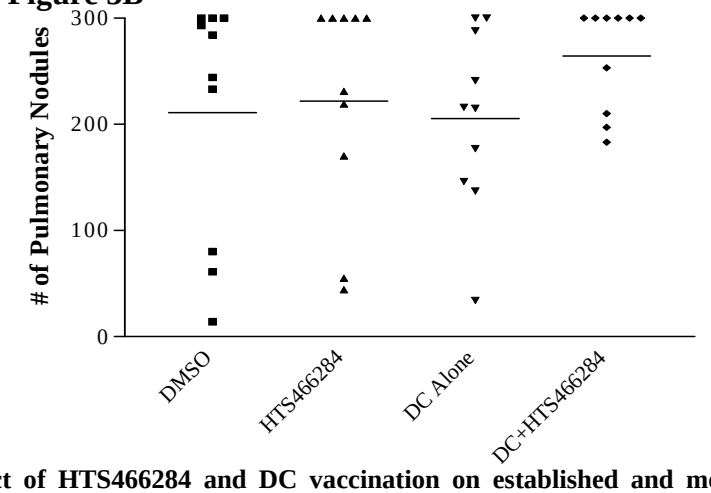

Figure 3 Effect of HTS466284 and DC vaccination on established and metastatic 4T1 tumors. Six-week-old female BALB/c mice were injected s.c. on day 0 with $5x10^4$ 4T1 cells into the mammary fat pad. On day 10 the mice were injected i.p. with 45 mg/kg of HTS466284 in 50 μl of DMSO daily for 19 days. Control animals received DMSO alone. Mice in the DC groups were injected s.c. with $1x10^6$ 4T1 tumor lysate pulsed, TNF-α matured DCs on days 12, 17, and 22. Lungs were collected at the end of the study, perfused with India ink, fixed in Fekete's Solution, and surface lung metastases were counted visually. The data represent: **A.** Mean tumor volume ± SEM of 10 individual mice and **B.** number of pulmonary nodules.

3. HTS466284 failed to enhance the ability of DC vaccines to treat 4T1 metastases in a residual disease setting.

Since most breast cancer related mortality is the result of disseminated disease, we wanted to evaluate if HTS466284 therapy can augment the ability of DC vaccination to treat residual metastatic disease. To this end, 4T1 tumors were established in Balb/c mice as described above. After 21 days when the tumors had reach an average of 97 mm^3, the tumors were surgically resected. The following day (day 22 post-tumor injection) the mice began receiving daily i.p. injections of 45mg/kg of HTS466284 and injections were continued until day 29 post-tumor injection. The mice were also injected s.c. with 10^6 4T1 tumor lysate-pulsed, TNF-α matured DCs on days 23 and 28. On day 30 the lungs were collected, stained with India ink as described previously, and surface lung metastases were counted visually. The data show that combination HTS466284+DC therapy had no significant effect on the growth of residual 4T1 pulmonary metastases (Figure 4).

Figure 4

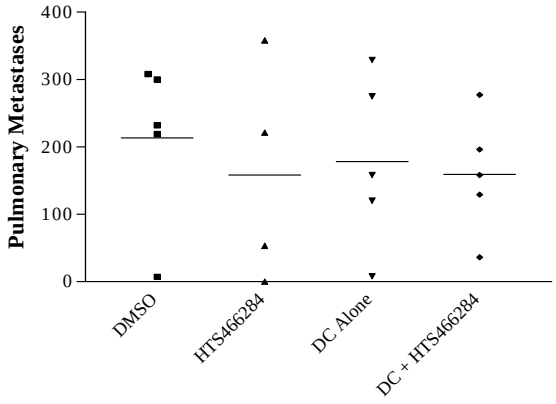

Figure 4 Effect of HTS466284 and DC vaccination on residual metastatic disease. Six-week-old female BALB/c mice were injected s.c. on day 0 with $5x10^4$ 4T1 cells into the mammary fat pad. On day 21 the tumors were surgically resected. The following day the mice were injected i.p. with 45 mg/kg of HTS466284 in 50 μl of DMSO daily for a total of 8 injections. Control animals received DMSO alone. Mice in the DC groups were injected s.c. with $1x10^6$ 4T1 tumor lysate pulsed, TNF-α matured DCs on days 23 and 28. On day 30 lungs were collected, perfused with India ink, fixed in Fekete's Solution, and surface lung metastases were counted visually. The data represent the number of pulmonary nodules from 5 individual mice.

4. SM16 inhibits TGF-β signaling events in 4T1 cells *in vitro* and 4T1 tumor tissue *in vivo*.
Since HTS466284 was unable to augment the efficacy of DC vaccination in the treatment of established and metastatic 4T1 tumors in our follow up established disease experiment and since this drug failed to improve the ability of DCs to treat 4T1 tumors in a residual disease setting, we decided to shift our focus from HTS466284 to an optimized TβRI kinase inhibitor, SM16 (2,3). Unlike HTS466284 which must be dissolved in organic solvents like DMSO, SM16 is readily soluble in aqueous solutions (2,3). In addition, SM16 can be delivered orally giving it more translational potential to human therapy (2,3). Before combining SM16 with DCs to treat established tumors we first wanted to demonstrate that this drug could inhibit TGF-β signaling events in our tumor model. Therefore, Smad2 phosphorylation was analyzed by western blot analysis in 4T1 cells treated with different concentrations of SM16 and exogenous recombinant human TGF-β_1. The data show that SM16 significantly blocks Smad2 phosphorylation in 4T1 tumor cells *in vitro* in a dose dependant manner with almost complete abrogation of TGF-β signaling events at a concentration of 2.5μg/ml (Figure 5A). We also evaluated the ability of SM16 to inhibit endogenous TGF-β signaling in established 4T1 tumors *in vivo*. To this end, mice bearing established 4T1 tumors were injected i.p. with either 10 or 40 mg/kg of SM16 and pSmad2 levels were analyzed by western blot in primary tumors and metastatic lung nodules. Controls received a single injection of cyclodextran (vehicle). The data show that a single dose of SM16 can profoundly suppress TGF-β signaling *in vivo* in both primary 4T1 tumors and lungs bearing metastatic 4T1 nodules (Figure 5B).

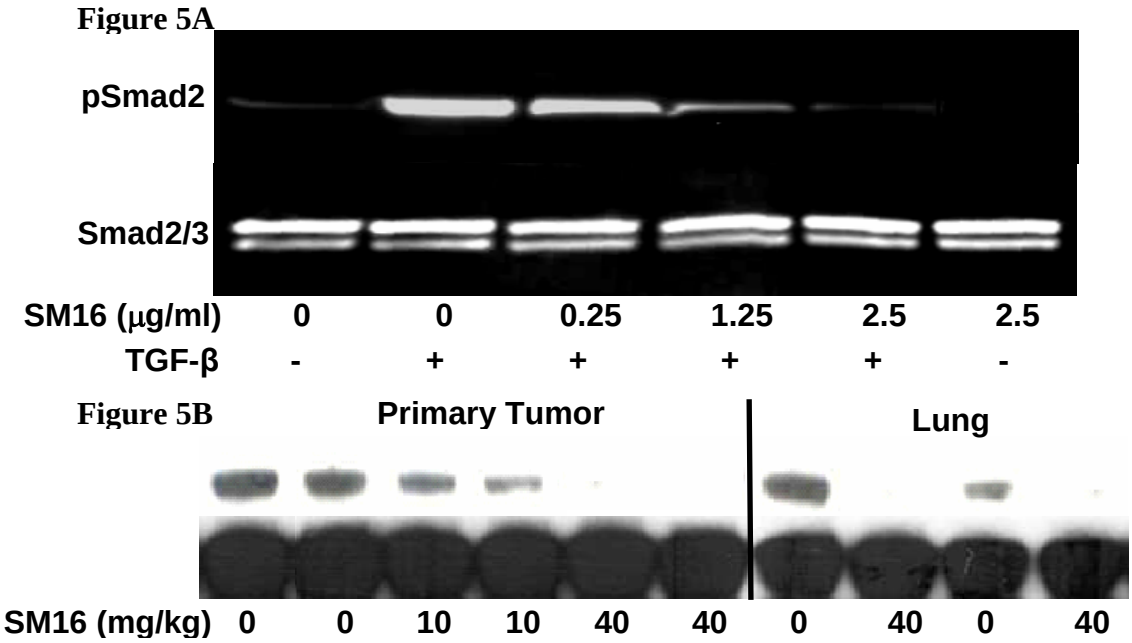

Figure 5 Effect of SM16 on TGF-β signaling in 4T1 cells *in vitro* and 4T1 tumors *in vivo*. A. 4T1 cells were treated with SM16 for 30 min followed by exposure to 2 ng/ml rhTGF-β_1 for 45 min. Cell lysates were prepared, resolved by 10% SDS-PAGE, and electrotransferred to PVDF membrane. pSmad2 was detected by incubating the membrane in rabbit anti-human pSmad2 antibody followed by HRP-conjugated anti-rabbit antibody. The membranes were then stripped and incubated with mouse anti-Smad2 antibody and membrane-bound total Smad2 was visualized with anti-mouse HRP secondary antibody. **B.** Mice bearing 21-day 4T1 tumors were injected i.p. with a single dose of SM16 (10 or 40mg/kg) or cyclodextran (vehicle control). For the lung metastases mice bearing 26 day 4T1 tumors were treated as described above. One hr. after treatment tissues were homogenized and proteins were resolved and visualized as described above.

5. SM16 inhibits 4T1 pulmonary metastasis formation.

In order to determine the optimal dose for SM16 therapy, mice bearing 10-day 4T1 tumors were treated i.p. with various concentrations of SM16 for 18 consecutive days. Controls were treated with cyclodextran (vehicle). Tumor growth was monitored and lungs were assessed for metastases 28 days post-tumor injection. The data show that SM16 had no significant effect on primary tumor growth at all concentrations tested (Figure 6A). However, SM16 at a dose of 40mg/kg significantly inhibited the formation of pulmonary 4T1 metastases compared to vehicle- treated controls ($P<0.05$) (Figure 5B). Animals treated with 40mg/kg of SM16 had an average of 31 pulmonary metastatic nodules compared to an average of 121 nodules in vehicle treated controls representing nearly a fourfold reduction in the number of lung metastases. Based on these findings the 40mg/kg dose was chosen for all future experiments.

Figure 5A

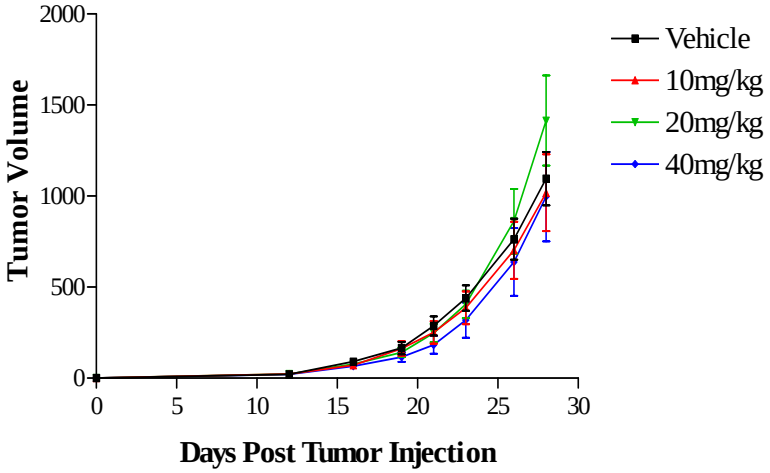

Figure 5B

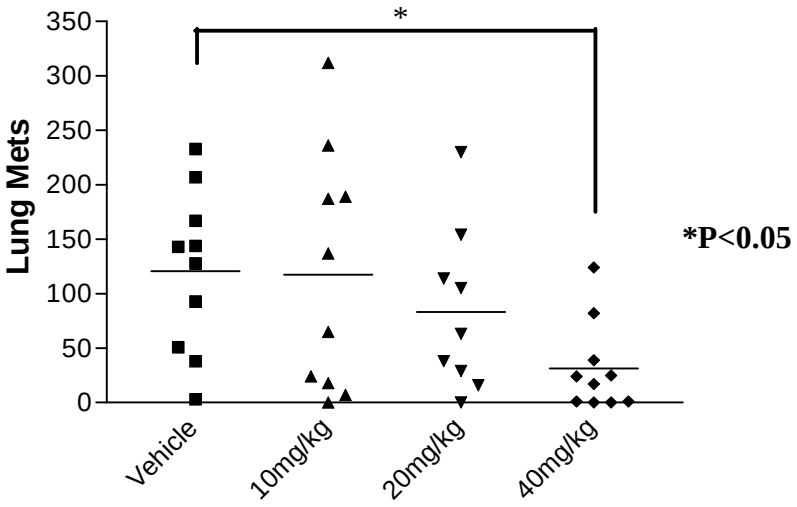

Figure 6. Effect of SM16 treatment on primary and metastatic 4T1 tumors. Six-week-old female Balb/c mice were injected s.c. on Day 0 with $5x10^4$ 4T1 cells into the mammary fat pad. On Day 10 the mice were injected i.p. with 10, 20, or 40 mg/kg of SM16. SM16 injections were continued daily until day 28 for a total of 19 injections. Control animals received cyclodextran (vehicle). Lungs were collected at the end of the study, perfused with India ink, fixed in Fekete's Solution, and surface lung metastases were counted visually. The data represent: **A.** Mean tumor volume ± SEM of 10 individual mice and **B.** the number of pulmonary nodules.

Key Accomplishments

1. Demonstrate the ability of HTS466284 to inhibit Smad2 phosphorylation in primary 4T1 tumors *in vivo*.

2. Demonstrate the inability of HTS466284 to consistently improve the efficacy of DC vaccines in the treatment of primary and metastatic 4T1 tumors.

3. Demonstrate the inability of HTS466284 to augment the ability of DC vaccines to treat residual 4T1 metastatic disease.

4. Demonstrate the ability of the improved TβRI inhibitor SM16 to suppress Smad2 phosphorylation in 4T1 tumor cells *in vitro* and in primary and metastatic 4T1 tumors *in vivo*.

5. Demonstrate the ability of SM16 to inhibit the growth of pulmonary 4T1 metastases *in vivo* at a dose of 40mg/kg.

For a detailed description of key accomplishment refer to the results section.

Reportable Outcomes

Presentations:

Matthew Rausch, Daruka Mahadevan, Xiamei Zhang, Leona Ling, and Emmanuel T. Akporiaye. Disruption of TGF-β Signaling Using Small Molecule TβRI Antagonists Improves the Efficacy of Dendritic Cell Vaccines. Poster, Second Annual Frontiers in Immunobiology & Immunopathogenesis Symposium. Tucson, Arizona. April 21, 2007.

Matthew Rausch, Daruka Mahadevan, and Emmanuel T. Akporiaye. Disruption of TGF-β Signaling Using Small Molecule TGF-β Receptor Type I Kinase Inhibitor Improves the Efficacy of Dendritic Cell Vaccines for Breast Cancer. Poster, 2006 Student Showcase. Tucson, Arizona. November10-11, 2006.

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3. Ling LE, Singh J, Chuaqui CE, et al. The use of virtual screening in ALK5 kinase inhibitor discovery and validation of orally active ALK5 kinase inhibitors in oncology. In: Jakowlew S, ed. Transforming growth factor-ß in cancer therapy. In: Teicher B, series ed. *Cancer Drug Discovery and Development*. Totowa: Humana Press. 2007.